Influence of Propylene Glycol on the Post Thaw Morphology of Buffalo Oocytes Vitrified dt different Stages in Vitro Maturation

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[Received:23Sept. 2014; Revised:1Oct.2014; Accepted: 1Oct 2014]

Abstract— The present experiment has been conducted to study the post thaw morphology of buffalo oocytes vitrified at different stages of in vitro maturation (IVM) using propylene glycol as a cryoprotectant. Cumulus oocyte complexes (COCs) obtained from slaughterhouse ovaries were randomly divided into 6 different groups: control (nonvitrified oocytes were matured for 24 h in maturation medium (MM) consists of TCM-199 supplemented with 10% w/v fetal calf serum (FCS) at 38±1°C and 5% C02 in a humidified atmosphere), 0 h (vitrified before the onset of maturation), 6,12,18 and 24 h groups (vitrified at 6, 12, 18 and 24 h, respectively, after the onset of maturation). Oocytes were exposed to vitrification solution (VS) consists of 40% w/v propylene glycol and 0.25 M trehalose in phosphate buffered saline (PBS) supplemented with 4% w/v bovine serum albumin (BSA) for 3 min at 20-25°C. Oocytes in VS were loaded into 0.25 ml French mini straw with 1M sucrose solution separated by two airspace on either side of VS. The straws were sealed with hot forceps and plunged directly into liquid nitrogen (LN2 -196°C). The straws were thawed after storage period of atleast 7 days by transferring them into a water bath at 37°C for 30 sec. The cryoprotectant was removed by exposing the oocytes to 1 M sucrose solution. Oocytes in 0, 6, 12, 18 and 24 h groups were further matured for additional 24, 18, 12, 6 and 0 h, respectively, to complete a total of 24 h maturation period. A sum of 495, 432, 457, 416 and 420 oocytes were vitrified in 0,6,12,18 and 24 h groups, respectively. After thawing, 444 (89.70%), 384 (88.89%), 418(91.47%), 381 (91-59%) and 387 (92.14%) oocytes were recovered in 0, 6, 12, 18 and 24 h groups, respectively. Recovered oocytes were examined to assess the post thaw morphology. Of which, 87.16 (387), 84.38 (324), 86.60 (362), 87.40 (333) and 90.96 (352) % of oocytes vitrified at 0,6,12,18 and 24 h groups, respectively, were found morphologically normal. From this study, it is clear that meiotic stages of oocytes do not influence the post thaw morphology in different vitrification groups.

Index Terms— Vitrification, In vitro maturation, Post thaw morphology and Buffalo Oocytes.

I. INTRODUCTION

Cryopreservation of oocytes at very low temperature (-196°C) is desired for both biological and commercial reasons. Cryopreservation can be done by conventional slow freezing and rapid freezing. Conventional freezing method is a tedious process, which involves many steps during freezing and thawing and needs technical skill and programmable freezing machine, which is costlier. An alternative approach to circumvent these problems is vitrification of oocytes introduced by Rall and Fahy, 1985 [1]. Vitrification is the solidification of liquid by extreme increase in viscosity during very rapid cooling [2]. The solid called glass has the molecular and ionic distributions of liquid state and thus avoids detrimental effects of extracellular and intracellular crystallization. Oocyte cryopreservation is reported to produce zona hardening that reduces the penetration of spermatozoa, thereby reducing the fertilization rate [3]. Zona hardening could be due to the premature release of cortical granules or some other mechanisms [4]. Zona hardening can be reduced or eliminated by adding serum or BSA to cryoprotectant solution [5, 6] which may bind to the active components in cortical granules and prevent modification of the structural proteins in the zona [7]. The technique of intracytoplasmic sperm injection (ICSI) can be used to overcome the problem associated with zona hardening following cryopreservation and results in fertilization rate near normal level [8]. The other gross morphological changes observed in zona after freezing and thawing are broken zona, single or multiple cracks and irregular shapes in the zona. Cryoprotectants are generally classified as 1. low molecular weight permeating agent (methanol, ethylene glycol, propylene glycol, dimethyl sulfoxide and glycerol), 2. Low molecular weight nonpermeating agent(glucose, sucrose and trehalose) and 3. High molecular weight nonpermeating agent (bovine serum albumin, polyvinyl alcohol and polyvinyl pyrrolidone) [9]. The present experiment has been conducted to assess the post thaw morphology of buffalo oocytes vitrified at the different stages of in vitro maturation using propylene glycol as a cryoprotectant.

II. MATERIALS AND METHODS

A. Materials

Chemicals

Sodium chloride (NaCl) and modified Dulbecco's phosphate buffered saline (mDPBS) used for ovary collection and washing were procured from Hi-Media, India. Tissue 1`culture medium-199 (TCM-199), antibiotics, antimycotics and other chemicals used for preparation of maturation, fertilization and embryo development media were of embryo culture or tissue culture grade, procured from Sigma Chemicals Co., USA. Propylene glycol and trehalose used for preparation of vitrification solution were also procured from Sigma Chemicals Co., USA.

Fetal calf serum was procured from Sigma Chemicals Co., USA and heat inactivated at 56°C for 30 min and stored at -20°C. Blood sample was collected from the estrus buffalo. The serum was separated, heat inactivated at 56°C for 30 min and stored at -20°C

All the media were filtered through membrane filter (0.22 µm) and stock solutions were kept at 4°C for maximum of 1 month. The working solutions were prepared and pre equilibrated 12 h before use.

Plasticware and glassware

All the plasticware used for culture viz., petriplates, culture dishes, multiwell dishes, centrifuge tubes and culture bottles etc. were purchased from Nunc: Denmark.

All the glassware (Borosil, India) used for culture were washed, packed and sterilized at 180°Cfor 1 h. Other than glassware, rest of culture accessories were autoclaved at 120°C and 15 psi for 30 min. All the plasticware and glassware were UV treated for 30 min before use.

B. Methods

Collection of ovaries

Buffalo ovaries were collected from local abattoir in sterile normal saline solution (NSS-0.89% w/v) supplemented with antibiotics (Penicillin G-100 lU/ml and streptomycin-100 µg/ml) and antimycotic (Amphotericin B - 2.5 µg/ml) at 30-35°C in an isothermic container and transported to the laboratory within 2 h of slaughter.

The surrounding tissues were trimmed off and the ovaries were washed several times with sterile NSS. The ovaries were exposed to 70% ethyl alcohol for 30 sec and finally washed in mDPBS.

Oocyte collection

Oocytes from surface follicles (>3 mm) of buffalo ovaries were collected by aspirating the follicles with 18 gauge needle attached to 5 ml syringe in oocyte collection medium (OCM). Contents from syringe were poured into a 50 ml test tube having OCM. Oocytes were allowed for gravitational settlement for atleast 15-20 min. Supernatant was then discarded and the remaining fluid was poured into a petridish containing OCM. Morphologically culturable oocytes i.e. those having compact, multilayered cumulus oocyte complexes (COCs) and evenly granulated cytoplasm were selected under stereo microscope (Bausch and Lamb, USA) and transferred to another petridish containing OCM. Finally COCs were washed 5 times in oocyte washing medium and 5 times in maturation medium.

In vitro maturation (IVM)

The COCs were randomly divided into 6 different groups with approximately equal number of oocytes.

Group I (Control)

The COCs were matured in MM for 24 h at 38±1°C and 5% C02 in humidified air.

Vitrification of oocytes

Vitrification solution (VS) consisted of propylene glycol (40% w/v) and trehalose (0.25 mol l-1) in phosphate buffered saline (PBS) supplemented with BSA (0.4% w/v).

Group II (Vitrification before onset of maturation)

The COCs immediately after collection (germinal vesicle stage) were exposed to VS at 20-25°C for 3 min. Then, the COCs in VS were loaded into 0.25 ml French mini straw (15-20 COCs in each straw) with sucrose (1 mol l ~1) solution separated by two air space on either side of VS. The straws were sealed with hot forceps and plunged directly into liquid nitrogen (LN2).

The straws were thawed after storage period of 7 days by transferring them into a water bath at 37°C for 30 sec. The cryoprotectant was removed by exposing the COCs to sucrose (1 mol¬- l Finally, the COCs were matured in MM for 24 h at 38±1 °C and 5% CO, in humidified air.

Group III (Vitrification at 6 h maturation)

The COCs were matured for 6 h and were vitrified, stored and thawed as in Group II. They were further matured for 18 h to complete 24 h maturation.

Group IV (Vitrification at 12 h maturation)

The COCs were matured for 12 h and were vitrified,

stored and thawed as in group II. They were further matured for 12 h to complete 24 h maturation.

Group V (Vitrification at 18 h maturation)

The COCs were matured for 18 h and were vitrified, stored and thawed as in group II. They were further matured for 6 h to complete 24 h maturation.

Group VI (Vitrification at 24 h maturation)

The COCs were matured for 24 h and were vitrified, stored and thawed as in group II. After thawing, oocytes from 0, 6, 12, 18 and 24 h were counted for post thaw recovery.

Assessment of morphology

Immediately after thawing, COCs in each group were examined under an inverted phase contrast microscope (Olympus, Japan) to assess the morphology.

Post thaw morphology of vitrified thawed COCs were made as per following criteria

Normal: Oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of the cellular content.

Damaged: Damaged oocytes were classified into crack in zona pellucida, splitting of oocytes, change in shape of oocytes and leakage of cellular content [10].

Statistical analysis

Statistical analysis was carried out by standard method described by Snedecor and Cochran (1994) [11].

III. RESULTS AND DISCUSSION

The recovered oocytes from all vitrification groups were examined under inverted phase contrast microscope to assess the morphology. Oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, vacuolization, degeneration or leakage of cellular contents were considered as normal.

From the recovered oocytes, 87.16, 84.38, 86.60, 87.40 and 90.96% of oocytes vitrified at 0, 6, 12, 18 and 24 h of maturation, respectively, were found morphologically normal. Remaining, 12.84, 15.62, 13.40, 12 60 and 9.04% of oocytes in 0, 6, 12, 18 and 24 h vitrification groups, respectively, were damaged during vitrification (Table 1).

Table 1: Impact of propylene glycol on morphological status of vitrified- thawed buffalo oocytes and types of damages (In parenthesis, percentage is shown)

Group	Number of recovered oocytes	Number of normal oocytes	Number of damaged oocytes	Types of damage			
				Crack in Zona pellucida	Splitting of oocytes	Change in shape	Leakage of cellular content
0 h	444	387 (87.16)	57 (12.84)	23 (40.35)	7 (12.28)	11 (19.30)	16 (28.07)
6 h	384	324 (84.38)	60 (15.62)	26 (43.33)	8 (13.35)	13 (21.67)	13 (21.67)
12h	418	362 (86.60)	56 (13.40)	23 (41.07)	9 (16.07)	9 (16.07)	15 (26.79)
18 h	381	333 (87.40)	48 (12.60)	21 (43.75)	7 (14.58)	9 (18.75)	11 (22.92)
24 h	387	352 (90.96)	35 (9.04)	15 (42.86)	5 (14.29)	6 (17.14)	9 (25.71)

Different types of damages have been observed in vitrified thawed oocytes like crack in zona pellucida, splitting of oocytes, change in shape and leakage of cellular content. Most commonly observed damage among all vitrification groups was crack in zona pellucida. 40.35% of oocytes in 0 h, 43.33% of oocytes in 6 h, 41.07% of oocytes in 12 h, 43.75% of oocytes in 18 h and 42.86% of oocytes in 24 h groups showed zona crack. Leakage of cellular content was noticed in 28.07, 21.67, 26.79, 22.92 and 25.71% of oocytes in 0, 6, 12, 18 and 24 h groups, respectively, which was next most commonly observed damage. In 0, 6, 12, 18 and 24 h groups, 19.30, 21.67, 16.07, 18.75 and 17.14% of oocytes showed abnormal shape which was third most commonly observed damage. Remaining, 12.28, 13.33, 16.07, 14.58 and 14.29% of oocytes in 0, 6, 12, 18 and 24 h groups, were of splitted type.

In the present study, 87.16, 84.38, 86-60, 87.40 and 90.96% of oocytes were morphologically normal when vitrified and thawed at 0, 6, 12, 18 and 24 h maturation, respectively. In this study, 9 -16% of the oocytes were damaged. They belonged to different categories like crack in zona pellucida, leakage of cellular content, change in the shape and splitting of oocytes. Similar kinds of damages were observed in earlier studies [10, 12-14].

Remarkable difference in the post thaw morphology was

not observed among the oocytes vitrified at different stages of maturation. Similar observation was made by Luna et al. [15] upon vitrifying bovine oocytes at different stages of maturation. They reported that 71.8, 65.5, 74.7 and 72.6% post thaw normality in bovine oocyte vitrified with 20% EG + 20% DMSO + 0.5 M sucrose at 0, 8, 12 and 24 h during IVM. Post thaw normality of immature buffalo oocytes varied between 88 and 98% even with different concentrations of cryoprotectant and different exposure times [10, 14]. Hong et al., [16] observed high (84%) post thaw normality of vitrified thawed immature human oocytes. But, post thaw normality of matured oocytes was reported to be higher (70-90%) [17 – 21] than immature oocytes (23-33%) [18, 22]. However, Martino et al., [23] observed low (34%) morphological normality in matured bovine oocytes. Variations in the post thaw morphological normality could be due to differences in types of cryoprotectant, concentrations of cryoprotectant, exposure time, stages of meiotic maturation and species of oocyte vitrified. Low molecular weight cryoprotectants such as propylene glycol protect the cells from solution effects by their collegative properties and also injury from ice crystals [24]. Low molecular weight nonpermeating cryoprotectant like trehalose exert the beneficial effect by causing cellular dehydration through changes in osmotic pressure [25].

IV. CONCLUSION

The above findings indicate that propylene glycol can be used as a cryoprotectant to preserve the oocytes at very low temperature (-196 o C).Further studies are needed to optimize the concentration of cryoprotectant and exposure time of oocytes to vitrification solution for better survivability after post thaw recovery.

V. ACKNOWLEDGEMENT

We are grateful to acknowledge IVRI, Izat Nagar and ICAR, New Delhi for the technical support and financial assistance rendered for this study.

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